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DNA Repair

Minireview

Eukaryotic mismatch repair: an update

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Abstract

The discovery that mutations in mismatch repair genes segregate with hereditary nonpolyposis colon cancer has awakened a great deal of interest in the study of the process of postreplicative mismatch repair. The characterisation of the principal players involved in this important metabolic pathway has been greatly facilitated by the amino acid sequence conservation among functional homologues of bacteria, yeast and mammals. The phenotypes of mismatch repair deficient mutants are also similar in many ways. In humans, mismatch repair malfunction demonstrates itself in the form of a mutator phenotype of the affected cells, an instability of microsatellite sequences and increased levels of somatic recombination. Moreover, mismatch repair deficient cells display also varying levels of tolerance to DNA damaging agents and are thought to be involved in the cell killing mediated by these agents. This article discusses some recent developments in this fast-moving field.

Keywords: Mismatch repair; Mutator phenotype; Hereditary nonpolyposis colon cancer; Drug resistance; Replication error

1. Introduction

Our understanding of the process of postreplicative mismatch correction has increased tremendously in the past five years, mainly due to the awakening of interest in this pathway linked to the discovery in 1993 that mutations in mismatch repair (MMR) genes segregate with a common form of an inherited cancer predisposition syndrome, hereditary nonpolyposis colon cancer (HNPCC). The field has been extensively reviewed in recent years and the interested reader is referred to a number of excellent articles on the subject that deal with MMR either globally [1–4], or only with specific topics such as MMR biochemistry [5,6], genetics [7] or the involvement of MMR in the toxicity of DNA damaging agents [8–10] or cancer [11–13]. The aim of the present minireview is to discuss the most recent developments in the field that pertain particularly to some 'extracurricular' activities of MMR proteins, especially those of the mismatch binding factors.

2. The prokaryotic model

The process of mismatch repair is highly conserved throughout evolution and this single fact has greatly facilitated its study in human cells. Most of our knowledge is based on seminal work with *Escherichia coli*, where the availability of mismatch repair deficient mutants has helped in the identifica-

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tion and the biochemical characterisation of the key polypeptides involved in this process. Our current picture of MMR in *E. coli* involves ten factors [14], which can be subdivided into two classes: those that are exclusively involved in postreplicative mismatch repair and those that participate also in other pathways of DNA metabolism. The former group consists of three polypeptides: MutS, MutH and MutL, while the latter contains MutU (UvrD), a DNA helicase II, Ssb, the bacterial single-stranded DNA binding protein, ExoI, a 3′-5′ exonuclease, RecJ and ExoVII, 5′-3′ exonucleases, DNA polymerase III holoenzyme and DNA ligase. These are involved in the following steps of the repair process. (i) The MutS homodimer binds the mismatch. (ii) In a series of steps that are chronologically ill-defined, the bound MutS homodimer recruits MutL (also in the form of a homodimer) and MutH to the repair complex, which then threads the DNA through itself in a bidirectional, ATP-dependent manner to generate a looped structure reminiscent of the Greek letter α or Ω, the so-called α-loop [15]. (iii) The three polypeptides sit at the base of the structure, whereby MutL activates the cryptic endonuclease activity of MutH. This protein then cleaves the DNA substrate in the unmethylated strand of the nearest hemimethylated GATC motif, which is the strand discrimination signal in this organism [16,17]. (iv) MutL helps to load the DNA helicase at this site [18–20], which proceeds to unwind the duplex preferentially towards the mismatch. (v) The exonuclease(s) degrades the displaced single-stranded DNA containing the mispair and (vi) the resulting single-stranded gap, stabilised by the Ssb protein, is filled-in by the polymerase III holoenzyme. (vii) The remaining nick is sealed by DNA ligase and (viii) the hemimethylated GATC site is modified by the Dam methylase to make the substrate refractory to further action by the MutHLS system. Although this working model is most likely correct, at least in a global sense, several questions remain to be answered. One of these concerns the directionality of the unwinding/degradation process. The efficiency of mismatch repair directed by a hemimethylated GATC site either upstream (5′) or downstream (3′) from the mispair is similar [21], despite the fact that UvrD helicase unwinds DNA preferentially in the 3′-5′ direction [18]. Is it possible that MutL loads UvrD only when the nick is 3′ from the mispair and that in the case of an upstream nick, the helicase activity associated with the pol III holoenzyme effects the repair process in a way analogous to nick translation? Maybe yes, but how does the enzyme complex know which helicase/exonuclease to load, i.e., which way to go? Is it possible that the directionality of the unwinding process is determined by the difference between the torsional stress in the loop and the flanking DNA sequences?

The other mystery concerns the role of MutL. Although clearly indispensable for repair, its biochemical role is unknown. It was described as a 'molecular matchmaker' that facilitates the interaction of DNA-bound MutS and MutH proteins, but the only experimental evidence that supported this hypothesis until recently came from an electron microscopic study that showed the three polypeptides situated at the base of the α-loop [15] and where MutL was shown to substantially accelerate the process of α-loop formation. The first hint as to what MutL might be actually doing during the repair process comes from the finding that the MutL N-terminus is structurally homologous to DNA gyrase and to class II DNA topoisomerases, as well as to the Hsp90 protein [22]. Correspondingly, MutL has a conserved ATP binding site and a weak ATPase activity, similar to Hsp90. These studies suggest that MutL may act as a molecular chaperone, aiding in the conformational changes that are most likely required to assemble a repair-proficient complex. from its individual components.

The third question concerns the size of the repair patch, which was determined by labelling and restriction analysis of the repaired heteroduplex [23,24] to span the region between the hemimethylated GATC site and a little distance past the position where the mispair had been. If the electron microscopic evidence [15] is right and the loop is symmetrical, such that the mismatch lies at its apex, how does the system know where to stop? Also here several explanations are possible [6], although all remain to be tested.

3. MMR in eukaryotes

Our current model of the MMR process in eukaryotic cells resembles that of *E. coli* to a great

extent, with two important differences. The first concerns the strand discrimination function. The hemimethylation status of newly replicated DNA appears to be used principally in gram-negative bacteria such as *E. coli* and *Salmonella typhimurium*. Already in micro-organisms such as *Streptococcus pneumoniae*, no MutH homologue has been identified and strand discrimination has been proposed to be mediated by the presence of strand discontinuities in the newly synthesized DNA [25]. In higher organisms, a similar mechanism may operate. Although the directionality of MMR was reported to be affected by the methylation status of the mismatch-containing DNA substrates in monkey cells [26], these results have to be interpreted with caution, because the substrates were mixed SV40 heteroduplexes carrying a 50/50 mixture of G/T and A/C mispairs with nicks either 5′ or 3′ from the mispair and/or with methylation either in the outer or the inner strand. In any case, MMR proceeds in a directional manner in organisms that do not methylate their DNA, such as *Saccharomyces cerevisiae* and *Drosophila melanogaster*. We therefore need to look elsewhere for strand discrimination signals. Biochemical experiments revealed that the directionality of the MMR process can be dictated in all systems (including *E. coli*) by nicks situated in the proximity of the mispairs [4]. Indeed, substrates containing such signals are efficiently corrected in a directional manner even in extracts of *E. coli* mutH mutants [4], implying that this factor is dispensable for mismatch correction per se. Although we still do not know how strand discrimination works in systems other than enterobacteria, the first hints as to the proteins involved came from the laboratory of Kunkel, where yeast two-hybrid studies identified PCNA as a MLH1-interacting protein [27] (abbreviations are MSH = MutS homologue, MLH = MutL homologue, PMS = post-meiotic segregation). PCNA has long been thought of solely as the factor that interacts with replicative and repair DNA polymerases and increases their processivity. However, in Kunkel's laboratory, PCNA was shown to be involved in a step prior to resynthesis of the repair patch [27], although it is clearly required in the resynthesis step, too [28], as would be expected from the finding that mismatch correction in human extracts requires polymerase-δ [29]. The evidence available to date suggests that the ring-shaped PCNA trimer links the polymerase with the MMR proteins, i.e., that they are an integral part of the replisome. Direct association of the MMR proteins with the replication fork would dispense with the need for specific strand discrimination signals and thus for proteins such as MutH, capable of recognising them. Needless to say that the strand discrimination problem arises only in the leading strand. Nicks or gaps between neighbouring Okazaki fragments could direct MMR in the lagging strand, and similar signals could be used in the resolution of mismatch-containing recombination intermediates. The apparent absence of a MutH homologue in most systems not only brings home the point that strand discrimination in *E. coli* is the exception rather than the rule. It confirms the findings obtained in in vitro mismatch repair experiments, which showed that covalently closed circular DNA heteroduplexes, or substrates where the distance between the mismatch and the nick is more than 2 Kb, are refractory to mismatch repair [30] and thus that the mammalian MMR system apparently lacks an endonuclease activity. This point is often forgotten during discussions of, for example, the role of MMR proteins in the processing of DNA damage other than base/base mismatches and insertion/deletion loops (IDLs).

The second fundamental difference is that the MutS and MutL functional homologues are heterodimeric rather than homodimeric. Thus, the mismatch binding function in human cells is mediated primarily by a heterodimer of two MutS homologues, hMSH2 and hMSH6, referred to as hMutSα [31], while the functional MutL homologue is a heterodimer of hMLH1 and hPMS2, named hMutLα [32]. (In yeast, the MutSα and MutLα factors consist of MSH2/MSH6 and MLH1/PMS1 polypeptides, respectively [7].) This means that the eukaryotic system has an inherent asymmetry. We shall now examine these factors in detail.

3.1. Eukaryotic mismatch binding proteins

An abundant factor, present in extracts of human cells and binding with high affinity to oligonucleotide substrates containing a G/T mismatch was first described in 1988 [33]. It was later shown to be composed of two MutS homologues, hMSH2 and

hMSH6 (also called GTBP or p160) [31,34]. Although the substrate specificity of this factor, frequently referred to as hMutSα, has not been extensively examined, it appears to bind with the highest affinity to oligonucleotide substrates containing G/T mispairs in many different sequence contexts, or IDLs of one nucleotide [35,36]. IDLs greater than one extrahelical nucleotide are also recognised by the factor, albeit here some flanking sequence effects are apparent [36,37]. In a recent study, IDLs in the context of microsatellite repeats were often bound more efficiently than similar loops in nonreiterated sequences [38]. The affinity of hMutSα for other types of base/base mismatches appears to be very low, at least as measured by the gel-shift assay. This contrasts with MutS, which forms high affinity complexes in this in vitro assay with G/T, A/C and G/G or A/A heteroduplexes (J.J., unpublished). These latter results agree with the findings of Su et al. [39], who also reported these base/base mispairs to be the preferred MutS substrates in a DNaseI footprinting assay. The affinity of MutS for a particular substrate appears to broadly reflect its correction efficiency in vitro and in vivo, at least in bacteria [40,41] and yeast [42,43], although some discrepancies will always be found. Our study examining the repair efficiency of various base/base mismatches in transfected CV1 cells [44] should not be considered here, as we were looking for short patch repair pathways and thus our substrates lacked the necessary strand discrimination signals.

In contrast to bacteria, human cells possess another factor, hMutSβ, composed of hMSH2 and hMSH3 [36,45], which has no appreciable affinity for base/base mispairs in vitro. It binds substrates with extrahelical nucleotides, whereby its affinity appears to increase with the size of the IDL, although flanking sequence effects were noted also in this case [36]. hMutSβ compensates for the loss of hMSH6 in loop repair, such that cells mutated in *hMSH6* do not display an instability of microsatellites consisting of dinucleotide repeats and larger [46]. Although hMutSβ can undoubtedly function in IDL repair, as shown by its ability to complement MMR deficient extracts [45,47,48], its role in the process under normal circumstances is uncertain. In most cell lines examined to date, hMutSα appears to predominate by a substantial margin [45,47] and repair of even large IDLs in extracts deficient in both hMutSα and hMutSβ (caused by a mutation in the *hMSH2* gene) could be complemented by the addition to the extracts of purified hMutSα [45]. Thus, although all three MutS homologues are expressed in human cells, hMutSα appears to play the predominant role in repair. This prediction is substantiated by the finding that although extracts of HCT15 cells, which lack hMSH6, contain around three-fold increased hMutSβ amounts [45], presumably because the expressed hMSH3 no longer has to compete for the available hMSH2 with hMSH6, these cells still have a mutation frequency more than 300-fold higher than HeLa [49]. Moreover, although the outcome of the competition for hMSH2 normally appears to favour hMSH6, overexpression of hMSH3, for example in cells where the *hMSH3* locus is co-amplified with the *DHFR* gene, does lead to a preferential formation of hMutSβ and the concomitant loss of base/base mismatch repair capacity [47,48]. Also in this case, the cells overexpressing hMutSβ, but lacking hMutSα, display a strong mutator phenotype [48]. However, it should be mentioned here that transfer of chromosome 5 into HHUA cells, which restores the expression of wild-type hMSH3 in this line, resulted in a considerable reduction of frameshift and missense mutations at the HPRT locus [50], implying that hMutSβ might address at least a subset of base/base mispairs in this cell line in vivo. The jury is therefore still out on this issue.

Both the heterodimeric mismatch binding factors have been purified to homogeneity from HeLa cells [31,35,45] or overexpressed in the baculovirus system [36,51]. As anticipated from the studies with the bacterial MutS proteins [52], the human homologues of MutS possess an intrinsic ATPase activity, which is required in the formation of the looped structure [15] and, presumably, also in the assembly of the repair complex. This activity was first described in 1992, when hMutSα (thought at that time to be composed of only one polypeptide, GTBP) was purified from HeLa cells [35]. It was noted that addition of ATP to the in vitro mismatch binding assay resulted in a dissociation of the protein from the oligonucleotide duplex. This reaction took place also in the absence of added magnesium, which suggested that ATP binding rather than hydrolysis was sufficient to mediate the dissociation. We observed no

effect of added ADP in the gel shift experiment, although, paradoxically, the affinity of the wild type hMutSα for the G/T mismatch was increased in the presence of AMP [35].

More recent studies, using the recombinant factor, substantially extended these findings. Gradia et al. [51] demonstrated that the hMutSα heterodimer behaves as a molecular switch, being ON (able to bind mismatched DNA) in the presence of ADP, but OFF (unable to do so) when complexed with ATP. This switch is most likely modulated by a conformational change of the heterodimer, which is induced by ATP binding and which does not require ATP hydrolysis. However, the protein does turn over ATP, albeit slowly [35,51,53], and this both in the presence and in the absence of DNA. Moreover, although it is likely that it is complexed with nucleotides in vivo, it should be pointed out that hMutSα can bind mispair-containing DNA in the absence of bound nucleotide [35,51,53]. Recombinant MutS [52] and homologues from yeast [54,55] and human [53] where the ATP binding site (the Walker type A motif) was mutated still bind to mismatch-containing substrates. The bacterial MutS becomes refractory to displacement by ATP, while the eukaryotic counterparts mutated in only one of the two subunits could still be dissociated from the substrate, albeit at higher ATP concentrations. Only the double mutants, where the ATP binding sites of both MSH2 and MSH6 had been mutated, become refractory to ATP-mediated displacement [53].

Although ATP hydrolysis is not necessary to displace hMutSα from oligonucleotide substrates in vitro, it is absolutely required for mismatch correction. In our in vitro assay, the double mutant was unable to complement mismatch repair deficient extracts, while the single mutants, modified either in the hMSH2 or in the hMSH6 ATP binding site, were able to do so, although not as effectively as the wild type protein [53]. These results contrast somewhat with experiments described by Alani et al., who demonstrated that overexpression of the mutant MSH2 in *S. cerevisiae* had a dominant negative effect [54]. The authors suggested that a similar situation could arise also in human cells with *hMSH2* mutations in the vicinity of the ATP binding site. Although this hypothesis cannot be disregarded, there exists also the possibility that the overexpressed protein, rather than associating solely with its cognate partner, MSH6, forms also other species, such as homodimers, which might bind DNA and interfere with the mismatch repair process. It should be noted here that both yeast and human MSH2 have been reported to bind DNA containing mismatches and IDLs [56–58]. The dominant negative phenotype of these mutants should therefore be examined in systems expressing quantities of the recombinant protein comparable with those of the wild type polypeptide.

As noted above, recombinant hMSH2 has been shown to bind to oligonucleotide substrates containing a variety of IDLs and base/base mismatches [57,58], as well as cisplatin-modified substrates [59] and Holliday junctions [60]. Unlike hMutSα, hMSH2 binds with a significantly higher affinity to substrates containing large or palindromic IDLs, rather than base/base mispairs [58] and this binding is ATP-insensitive [58]. This latter point, coupled with the finding that in cells, hMSH2 is largely complexed with hMSH6 or hMSH3 [31,34,61], has brought into question the biological relevance of these studies. However, these experiments should not be disregarded, as the results may simply reflect the role of hMSH2 in the context of the heterodimer(s). It is conceivable that this polypeptide anchors the complex to homoduplex DNA, while the other subunit, be it hMSH6 or hMSH3, ensures its substrate specificity. It is interesting to note in this respect that when short (30–40 mer) oligonucleotide probes were used in DNA binding studies with hMutSα, ATP-sensitive, mismatch-specific complexes were formed [35]. UV irradiation of these mixtures resulted in cross-linking of the DNA to only hMSH6 [33,53]. However, when longer probes were employed, a nonspecific complex was formed in addition to the specific one, whereby the former was no longer responsive to ATP [51]. Although the nonspecific complex could be due to aggregation of the heterodimer on the DNA, its uniform, albeit slow, electrophoretic mobility more likely reflects either a DNA/hMutSα complex where the protein or the DNA molecule have undergone an ATP-driven conformational change [55], or where the protein binds as a heterotetramer.

The yeast MSH2 and MutSα have also been reported to bind to Holliday junctions with sub-

nanomolar affinity [60,62]. Also in this case, the biological significance of the results was questioned, as these structures are clearly substrates for highly specialised recombinases and resolvases and there appears to be no a priori reason why they should be bound by MMR proteins. However, MMR proteins influence the fidelity of homologous and homeologous recombination events, inasmuch as greater sequence divergence between the recombining molecules is permitted during recombination in MMR-deficient cells [3,7,63]. By binding to regions of sequence heterology, MMR proteins could therefore signal to the junction-specific resolvase enzymes when to stop branch migration by cleaving the crossover. The junction bound by MutSα was indeed shown to be more efficiently cleaved by junction-resolving enzymes T4 endo VII and T7 endo I [62]. Moreover, resolution of the junction will generate a specific nick in each heteroduplex, which might serve as an entry point for MMR proteins responsible for removing any eventual mismatches from the recombined molecules.

3.2. MutL homologues

Like the MutS family, the eukaryotic MutL homologues are also highly conserved, especially at their N-termini [64]. Purification of hMutLα, a complex of hMLH1 and hPMS2, from HeLa cells [32] implies that the yeast equivalent, MutLα, is also a heterodimer, this time of MLH1 and PMS1. Although no biochemical characterisation of the proteins purified from *S. cerevisiae* has been carried out to date, there is evidence that the two polypeptides, synthesized in an in vitro transcription/translation system, interact with one another [65].

The human hMutLα heterodimer purified from HeLa cells could be shown to complement mismatch repair deficient extracts from cells with mutations in either the *hMLH1* or the *hPMS2* genes [32]. The heterodimer produced in the baculovirus system is equally active in the in vitro mismatch repair assays (M. Räschle, G. Marra and J.J., manuscript in preparation). These latter studies confirm that the functional MutL homologue in human cells is the heterodimer of hMLH1 and hPMS2. This is an important point, as a third human MutL homologue, hPMS1, was shown to be mutated in one HNPCC family [66] and was also suggested to be involved in mismatch repair. Although we could show that hMLH1 and hPMS1 do indeed form a heterodimer (M. Räschle, G. Marra and J.J., manuscript in preparation), we have so far been unable to assign a biochemical function to this complex.

As in the case of the bacterial protein, we do not know at this time what role MutLα plays in the repair process. It is likely that it has a chaperone function as discussed for MutL above. Moreover, as already mentioned, due to the fact that hMLH1 was shown to interact with PCNA [27], it is possible that this complex functions in strand discrimination in eukaryotes [6,27]. The asymmetry of hMutLα might also dictate the directionality of the repair process on the DNA. It is conceivable that hMLH1 might interact with helicases and/or exonucleases that process the heteroduplex from the 3′ nick, while hPMS2 interacts with the repair factors situated 5′ from the mismatch, such as exonuclease I, which appears to be implicated in MMR in *S. cerevisiae* [67]. This prediction is based on the findings that certain cell lines mutated in the *hMLH1* gene are proficient in the processing of heteroduplexes from a 5′ nick, but not from an equivalent signal positioned downstream from the mispair [68], while the opposite appears to be true for some *hPMS2* mutants [69].

The biochemistry of MMR in human cells is currently the subject of intense study in a number of laboratories. The involvement of polymerase-δ [29] and RPA [70], homologues of the bacterial pol III and Ssb, respectively, has already been documented, and it is to be expected that the other missing members of the repairosome will be identified in the near future. Coupled with the powerful genetics of *S. cerevisiae*, which has been instrumental to many of the discoveries in the field [7], and with recent attempts at crystallisation of the MMR proteins, there is no doubt that also the finer mechanistic points of the process will be elucidated soon. However, rather than speculating on these, I have decided to devote the remainder of this article to discussion of the roles of the MMR proteins in the processing of damage not associated with DNA replication. As already mentioned, cells deficient in mismatch repair have been reported to be tolerant to treatment with alkylating agents [8], as well as to some bulky chemicals such as cisplatin [71], and to ionising

radiation [72]. In all these instances, active MMR contributes to cell killing, rather than exerting a protective effect by assisting in the removal of the damage from DNA. As it is of relevance to cancer therapy, the elucidation of the molecular mechanisms underlying this phenomenon has received much attention recently.

4. Mismatch repair deficiency and DNA damage tolerance

The involvement of the MMR system in the toxicity of alkylating agents was first noted in *E. coli*, where the hypersensitivity of a *dam*(−) strain to methyl nitrosourea (MNU) was rescued by secondary mutations in the *mutS* or *mutL* genes [73]. A similar effect was observed several years later in MMR-deficient human tumour cell lines [74]. This phenotype was described as alkylation tolerance rather than resistance, because the cells were able to tolerate the presence of a high number of modified bases in their DNA, which persisted and were mutagenic, but which were not toxic to them. The number of DNA modifying agents which are tolerated, to a greater of smaller extent, by MMR-deficient cells has increased from simple alkylating agents such as MNU, MNNG, procarbazine and temozolomide to busulfan, mercaptopurine, 6-thioguanine, carboplatin, cisplatin, doxorubicin, etoposide (see Refs. [8,10,75] for reviews) and ionizing radiation [72]. As this subject has been reviewed only very recently, I shall attempt to discuss here only the mechanistic aspects of the phenomenon. In the case of the methylating agents, the killing lesion was identified as 6-*O*-methylguanine (6 MeG), because MMR deficiency rescues killing predominantly in cells expressing low amounts of the specific 6 MeG repair enzyme, methylguanine methyltransferase (MGMT) [8]. It was suggested that in the absence of MGMT, 6 MeG persists in DNA until replication, where the DNA polymerase attempts to match it with a complementary nucleotide. Because 6 MeG forms an imperfect base pair with both C and T, the mismatch repair system detects both these 'mispairs' [76,77] and initiates the process of mismatch correction. During the resynthesis step, the polymerase fails again to find an ideal partner and the repair process is reinitiated [75]. This replication/repair ad nauseam has two possible consequences. As it would effectively block the progress of the replication fork, it is conceivable that such 'futile repair' will be interpreted as a signal to the apoptotic machinery that the cell should undergo programmed death. The second alternative is that the 6 MeG in the template strand will bring about chain termination and dissociation of DNA polymerase, with DNA synthesis resuming behind the damage from a new primase-initiated site. This would result in a single strand gap opposite the modified guanine. The killing effect of the lesion would come during the following cycle of DNA replication, when the replication fork arrives at a discontinuity and thus generates a double-strand break. At this time, there is scant evidence for either mechanism. The apoptotic signalling hypothesis is supported by the finding [78] that treatment of an MMR proficient lymphocytic line TK6 with temozolomide resulted in the induction of p53 and p21, while no such induction was observed in alkylation-tolerant MT1 cells, which were derived from TK6 and are known to be MMR-deficient due to mutations in both alleles of the *hMSH6* gene [46]. Importantly, p53 is functional in MT1 cells, as etoposide treatment induced p53 and p21 in both MT1 and TK6 lines with similar kinetics [78]. Although these data might appear to implicate p53 in inducing apoptosis in the treated cells as a result of signalling through the MMR proteins, there are clearly more sides to this story, because other cell lines with mutated p53 display sensitivity to alkylating agents similar to TK6. Moreover, tolerant, MMR-deficient lines can be sensitised to alkylating agents by the re-introduction of a wild type copy of the mutated mismatch repair gene, without the need to revert also their pre-existing p53 mutation (see below). However, MMR proteins do appear to be able to signal to the cell cycle at the G_2/M phase, as alkylating agents bring about a checkpoint arrest at this stage in mismatch repair proficient, but not MMR-deficient cells [79].

Evidence in favour of the double-strand break-associated killing is also indirect, but it has been noted in several laboratories that the cytotoxicity of methylating agents comes about primarily during the second S-phase [80] following treatment.

Tolerance to bulky DNA modifying agents such as cisplatin falls into a different category. Cisplatin modifies DNA by forming inter- and intrastrand cross-links between adjacent guanines, as well as covalently linking DNA to proteins. These bulky lesions should be removed by the nucleotide excision repair (NER) pathway and it was therefore somewhat surprising to discover that MMR-deficient cells are around two-fold more resistant to these agents [81,82]. This low level of resistance is probably due to the fact that although the cisplatin lesions are indeed primarily removed by NER, those that persist might be detected by the MMR system during DNA replication. Such persistent lesions could be 1,2-GpG intrastrand cross-links, which appear to be removed from modified DNA with the lowest efficiency by the NER system [83] and which have been shown to be bound by hMutSα in vitro [68]. However, in deference to methylation damage, cisplatin DNA modifications are replication-blocking lesions. In bacteria, cisplatin treatment induces the SOS response and error-prone replication by-pass. Mammalian cells appear to lack an efficient SOS response, but survive probably due to a recombination-mediated by-pass or a similar mechanism. One role of MMR proteins in cisplatin toxicity might therefore be simply that of signalling to the apoptotic machinery from a stalled replication fork. In their absence, the polymerase might by-pass the lesion, either in an error-prone manner, or by re-priming downstream. In the case of an error-prone by-pass, MMR proteins might initiate a repair process that could also result in cell killing, in a way similar to that described above for alkylating agents. It could be shown that oligonucleotides containing a single 1,2-GpG intrastrand cross-link paired with a CpC in the complementary strand are worse substrates for hMutSα than when the lesion is base-paired with a CpT, which corresponds to at least one of the possible products of an error-prone by-pass event [84].

Despite the available evidence, it is unclear at present how MMR deficiency helps overcome cisplatin toxicity. The fact remains that more cells survive the treatment, which means that more cells must have somehow by-passed the damage. Experiments aimed at elucidating the process underlying this survival mechanism are currently the subject of intensive study in several laboratories.

MMR deficient cells were also reported to respond differently than their MMR-proficient counterparts to ionizing radiation (IR). In one study [72], mouse Msh2, Mlh1 and Pms2 KO lines were shown to be more resistant to IR than the respective isogenic controls. This effect was small, but reproducible and suggested that the mismatch repair proteins might be implicated also in the cytotoxicity associated with damage caused by active oxygen species. The small difference between the MMR proficient and deficient cells could be linked to the fact that ionizing radiation induces a plethora of structurally diverse damage, which is addressed by several repair pathways and that only a subset of the DNA lesions are linked with MMR, either through damage signalling and apoptosis, or by direct repair. The involvement of MMR in apoptosis has been discussed in relation to alkylating agents and hMLH1 was described to restore also the IR-induced G2/M checkpoint in HCT116 cells complemented with chromosome 3, as well as in matched mouse Mlh1 $-/-$ and $+/+$ cells [85]. In deference to the study from Glazer's laboratory [72], the MMR-deficient cells in the latter study were reported to be more sensitive to IR than their MMR-proficient counterparts. This discrepancy requires clarification.

Regarding the role of MMR in the repair of IR damage, a recent study from Crouse's laboratory suggests that the mismatch repair system might play a much more decisive role than was thought up to now, inasmuch as the mutator phenotype of MMR deficient *S. cerevisiae* mutants, which is up to 1000-fold above wild type under normal growth conditions, was lowered up to 60-fold when the strains were grown anaerobically (G. Crouse, personal communication). It is interesting to note in this respect that MMR deficiency appears to abolish transcription-coupled nucleotide excision repair [86], and that thymine glycols, which are produced in large amounts by IR, have recently been shown to be addressed by TCR [87] in a manner which depends on the MMR system.

5. Complementation of mismatch repair deficiency

As already mentioned, extracts of mismatch repair deficient human cell lines could be made mismatch

repair proficient by complementation with purified hMutSα and/or hMutLα. However, as this in vitro assay can measure the efficiency of complementation of only one aspect of the multifaceted phenotype of MMR deficient cells, it was necessary to undertake gene transfer experiments in order to ascertain that expression of the wild type copy of the mutated gene is sufficient to correct also the microsatellite instability (MSI), cell cycle and DNA damage tolerance phenotypes. The first successful experiments in this field were carried out in Boland's laboratory, where the *hMLH1* defect of HCT116 cells was corrected by the transfer of chromosome 3 [88]. Later studies succeeded in transferring chromosome 2 to HEC59 cells, mutated in *hMSH2*, as well as to HCT15 and DLD1 lines with a mutation in the *hMSH6* gene [89]. All these experiments reverted the MSI and spontaneous mutation frequencies to levels expected for MMR proficient cells. The alkylation tolerance trend was also reversed and mismatch repair was restored. Transfer of chromosome 5 to HHUA cells, which carry mutations in the hMSH3 and hMSH6 genes resulted primarily in the restoration of IDL repair, although the frequency of substitution mutations was also lowered somewhat [90]. All these reports pay witness to the fact that an MMR defect can be complemented by the expression of a wild type copy of the mutated protein from its natural environment on its cognate chromosome. However, these experiments also had a shortcoming, in that the phenotypic correction achieved by the transfer of entire chromosomes could have been due to expression of other genes located on that same chromosome. Although such a coincidence is highly unlikely, it should not be disregarded, particularly because chromosome 2 carries both the *hMSH2* and *hMSH6* genes and chromosome 3 contains the *hMLH1* and *hPMS1* genes. One way of eliminating doubts regarding these experiments is to correct the phenotypes by expression of single genes.

Until recently, attempts to express cDNAs of MMR genes in mismatch repair deficient lines met with little success, for reasons that are not clear. However, Risinger et al. have now reported that expression of hPMS2 cDNA in HEC-1-A cells resulted in the restoration of IDL repair capacity. The surprising finding that the correction of base/base mispairs was not restored could be explained when the authors identified a mutation also in the *hMSH6* gene in this line [91]. The fact that the HEC-1-A line is a double-mutant was discovered independently also in our laboratory, where complementation of its extracts with recombinant hMutLα resulted in the correction of only IDL substrates, while full mismatch repair proficiency was restored only upon the addition of both hMutSα and hMutLα (G. Marra, I. Iaccarino, J.J., unpublished).

Our attempts to express hMSH6 cDNA in HCT15 cells resulted also in only a partial correction of the phenotype, although the reason underlying this phenomenon was different from the study of Risinger et al. [91]. In our case, proficient expression of the hMSH6 mRNA was not accompanied by a corresponding elevation in the intracellular levels of the protein. One explanation for this difference could be the fact that this cell line has compensated for the lack of hMSH6 by overexpressing hMSH3. The levels of hMutSβ in HCT15 extracts have been found to be about three-fold higher than in HeLa cells [45]. As mentioned above, hMSH6 has to compete for hMSH2 with hMSH3, and the outcome of this competition under normal circumstances appears to favour the formation of hMutSα. The elevated hMSH3 levels in HCT15 cells will affect the outcome of this equilibrium, as described above for the methotrexate-resistant lines [47,48], such that a substantial amount of the vector-encoded hMSH6 may fail to find its cognate partner (hMSH2) and be degraded.

Overexpression of hMLH1 in HCT116 cells was also reported, although the authors studied only its effect on cell growth rather than on the mutator phenotype of the transfectants [92].

6. Relevance of mismatch repair deficiency to human cancer

Interest in the study of MMR was greatly enhanced in 1993, when the first locus of HNPCC was identified on chromosome 2p15-16 [93]. The underlying reason for this interest was the finding that tumours of HNPCC patients demonstrated a substantially greater instability of microsatellite markers than the corresponding normal tissue from the same individuals. This implied that HNPCC tumours had ei-

ther lost the ability to replicate these sequences with high fidelity, or, alternatively, that they were unable to correct IDL mismatches generated during the replication through these repeated motifs [94]. The association of HNPCC with an MMR deficiency was confirmed only nine months later, when the 2p15-16 locus was shown to house the *hMSH2* gene [95,96]. Subsequent studies could show that cell lines with MSI were MMR-deficient [97] and mutations in the *hMSH2* and *hMLH1* genes have been shown to segregate with around half of the HNPCC families. As this topic has been extensively reviewed in the past years [1,2,11,13], I shall only mention the most recent developments here.

Although early reports identified families with mutations in *hPMS2* and *hPMS1* genes [98], these examples remain isolated. However, the involvement of the former gene in tumourigenesis could be substantiated in knock-out mouse models, where *Pms2*-null mice were found to be tumour-prone. The *Pms1* knock-out mice are so far largely free of malignancies [99]. Coupled with the fact that we have to date no biochemical role for the PMS1 protein, we must await the results of further studies before we understand what—if anything—PMS1 does in MMR and/or in tumourigenesis. The answer to the question why mutations in *hPMS2* are so rare when compared to *hMLH1* mutations is unclear at present. The products of the two genes function together in the hMutLα heterodimer and cell lines lacking either protein display similar phenotypes. Interestingly, *Pms2* mutations display a different phenotype also in animal models. Mice lacking this gene have a high tumour incidence, but, unlike *Msh2* or *Mlh1* KO mice, do not have intestinal cancers; the animals succumb principally to lymphomas [99]. The reasons underlying these differences are unclear at present.

Although mutations in the *hMSH6* gene have been identified only very recently in atypical HNPCC families [100], Msh6 KO mice are cancer-prone even in the presence of a fully functional *Msh3* gene [101]. This confirms the prediction that Msh3 cannot compensate for Msh6. Thus, the reason why more HNPCC families with mutations in the *hMSH6* gene have not been identified could lie either in the fact that the hMSH6 $-/-$ tumours display only a very limited MSI and therefore that these families have been excluded from the screening programs, or that the *hMSH6* gene is less susceptible to mutation than its MMR counterparts. The latter statement is most likely incorrect, as mutations in the C_8 frameshift hotspot of *hMSH6* have been detected in a number of sporadic colon tumours [102]. It is therefore likely that more HNPCC families will be shown to segregate with *hMSH6* mutations, providing that they are suitably pre-selected.

One issue that has been raised in relation to the mouse models of HNPCC was the predominance of lymphomas over intestinal dysplasias in these animals. This should not be too surprising, as these animals are homozygous mutants from the point of conception, whereas HNPCC kindreds are heterozygous for a given mutation. Thus, tumours in the KO mice will tend to arise in rapidly proliferating tissues where the mutator phenotype might be exacerbated by increased recombination or other processes, such as apoptosis, that involve MMR proteins. In HNPCC kindreds, who are globally MMR proficient, the tumours will arise in tissues that are most likely to acquire the 'second hit' which will inactivate the wild type MMR gene allele. Moreover, mutations in other genes predisposing to colonic malignancy or mutators that modify this tissue will also play an important role. This hypothesis was substantiated by recent experiments, where *Pms2* $-/-$ mice, which are normally not prone to intestinal tumours [99] displayed elevated rates of colonic tumours upon crossing with the *Min* mouse, deficient in the APC tumour suppressor gene [103]. These results might implicate dietary factors and mutagens as playing an important role in the puzzling tissue specificity of HNPCC tumours, which display an uncanny preference for the epithelium of the colon, ovary and endometrium with the exclusion of most other tissues. The role of mutagens in the carcinogenesis of MMR-deficient mice was demonstrated in animals lacking Msh2, which displayed an elevated tumour formation following treatment with carcinogens such as EMS [104].

MSI has been identified in an ever-increasing number of sporadic tumours of the colon and other tissues [102,105]. In these tumours, mutations in the *hMSH3* and *hMSH6* genes appear relatively frequently, but they occur in A_8 and C_8 mononucleotide runs, respectively [105]. Because these microsatellite mutations are signatures of MMR-defi-

cient cells, it would seem probable that they resulted in tumour cells which have already lost mismatch repair. In sporadic tumours, it is rather unlikely that two copies of an MMR gene would have been inactivated through random mutagenesis. However, colonic polyps and adenomas often display aberrant patterns of cytosine methylation and elevated levels of expression of the cytosine DNA methyl transferase DNMT [106]. Correspondingly, a number of genes in these growths are inactivated through epigenetic silencing. One such gene is *hMLH1*; its promoter is a CpG island that has been shown to be hypermethylated in several MMR-deficient tumour cell lines and sporadic tumours [107,108]. More extensive studies seem to indicate that this gene is methylated in a majority of sporadic tumours with MSI (R. Kolodner, personal communication). Future experiments will tell whether other MMR genes are subject to similar fate. Of great importance is to establish whether cytosine methylation plays a role also in HNPCC tumourigenesis, as numerous studies have failed to find the mutation inactivating the wild type allele of the MMR gene that segregates with the disease (HNPCC Database).

7. Other roles of mismatch repair proteins

As noted above, MMR proteins are implicated not only in postreplicative mismatch correction, but also in cell killing mediated by DNA modifying agents. However, recent literature describes the involvement of MMR also in other processes of DNA metabolism. One of these is meiotic recombination, where evidence from both *S. cerevisiae* and transgenic mouse model systems indicates that the MutL homologues might have also separate functions. Thus, MLH1 is the only *S. cerevisiae* MMR homologue that is epistatic with MSH4 and MSH5 during meiotic crossing over [109,110]. The latter two MutS homologues therefore probably substitute for MSH2, 3 and 6 in meiosis. A similar situation may arise also in mice. While both sexes of *Mlh1* KO mice are sterile, only the female *Pms2* KO mice are similarly affected and there are no documented fertility problems in the *Msh2* KO animals [99]. Deficiency of hPMS2 was also reported to affect the rates of somatic recombination in human cells [111]. This evidence implies that MMR proteins are involved also in the control of homologous recombination fidelity, a prediction made some time ago by Radman et al. [112] and supported by an increasing number of papers which demonstrate either the involvement of MutS or MutL in RecA-mediated branch migration [113,114], or of their eukaryotic homologues in the binding to Holliday junctions [60,62] and in recombination in vivo [115–117].

8. Conclusions

Mismatch repair is an extremely complex, but also an extremely rewarding field of study. Its link with cancer makes our efforts directly relevant to human health, and it is hoped that developments in this field will lead to better diagnosis, and thus also to better prognosis of HNPCC patients and sporadic cancer patients with MMR deficient tumours. It is hoped that our intimate knowledge of the MMR mechanism will help in the identification of new points of therapeutic intervention and perhaps even prevention.

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